A Simple Plasma Retinol Isotope Ratio Method for Estimating β-Carotene Relative Bioefficacy in Humans: Validation Using Model-Based Compartmental Analysis [1-3]

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Names for PubMed listing: Ford JL, Green JB, Lietz G, Oxley A, Green MH

Word count: 6,594

Number of figures: 6

Number of tables: 1

OSM Submitted: Supplemental Table 1, Supplemental Methods 1 and 2, Supplemental WinSAAM Deck

Running title: Prediction of β-Carotene Bioefficacy

[1] Sources of support: Partial support for this work was provided by the College of Health and Human Development, The Pennsylvania State University.

[2] Author disclosures: J.L. Ford, J.B. Green, G. Lietz, A. Oxley, and M.H. Green, no conflicts of interest.

[3] Supplemental Table 1, Supplemental Methods 1 and 2, and Supplemental WinSAAM Deck will be available at the “Online Supporting Material” link in the online posting of the article and from the same link in the online table of contents at jn.nutrition.org.

[6] Abbreviations used: AUC_p, area under the plasma retinol isotope response curve; DT, delay time; FD_p, fraction of dose in plasma; IRM, isotope reference method; L(I,J), fractional transfer coefficient; RIR, plasma retinol isotope ratio.

Abstract

Background: Provitamin A carotenoids are a significant source of dietary vitamin A for many populations. Thus, accurate and simple methods for estimating carotenoid bioefficacy are needed to evaluate the vitamin A value of test solutions and plant sources. β-Carotene bioefficacy is often estimated from the ratio of areas under plasma isotope response curves after subjects ingest labeled β-carotene and a labeled retinyl acetate reference dose (isotope reference method; IRM) but, to our knowledge, the method has not yet been evaluated for accuracy.

Objective: Objectives were to (1) develop and test a physiologically-based compartmental model that includes both absorptive and post-absorptive β-carotene bioconversion and (2) use the model to evaluate the accuracy of the IRM and a simple plasma retinol isotope ratio (RIR; labeled β-carotene-derived retinol / labeled reference dose-derived retinol in one plasma sample) for estimating relative bioefficacy.

Methods: We used model-based compartmental analysis (Simulation, Analysis and Modeling software) to develop and apply a model that provided known values for β-carotene bioefficacy. Theoretical data for ten subjects were generated by the model and used to determine bioefficacy by RIR and IRM; predictions were compared to known values. We also applied RIR and IRM to previously-published data.

Results: Plasma RIR accurately predicted β-carotene relative bioefficacy at 14 d or later. IRM also accurately predicted bioefficacy by 14 d, except that, when there was substantial post-absorptive bioconversion, IRM underestimated bioefficacy. Based on our model, 1 d predictions of relative bioefficacy include absorptive plus a portion of early post-absorptive conversion.

Conclusion: The plasma RIR is a simple tracer method that accurately predicts β-carotene relative bioefficacy based on analysis of one blood sample obtained at 14 d or longer after co-ingestion of labeled β-carotene and retinyl acetate. The method also provides information about

the contributions of absorptive and post-absorptive conversion to total bioefficacy if an additional sample is taken at 1 d.

Key words: β-carotene; bioconversion; bioefficacy; carotenoids; humans; isotope reference method; model-based compartmental analysis; retinoids; retinol isotope ratio; WinSAAM

Introduction

Provitamin A carotenoids, especially β-carotene, are an important dietary source of vitamin A, especially in low-income countries where vitamin A deficiency remains a public health problem (1). Available data, based mainly on results for β-carotene, indicate that the bioavailability and bioconversion of provitamin A carotenoids to vitamin A [i.e., bioefficacy (2)] are affected by many factors, including food matrix, preparation methods, genetics, age, etc. (3). Although several methods have been proposed and used to measure β-carotene bioefficacy (4, 5), none are ideal for use in the field; in addition, none have been verified by comparison to known reference values. Researchers need a simple, accurate, and feasible method to assess bioefficacy because of β-carotene's contribution to whole-body vitamin A stores and plasma retinol homeostasis.

Recently (6), we used model-based compartmental analysis to study plasma retinol kinetics and to estimate relative bioefficacy of β-carotene in healthy adults who had consumed stable isotope-labeled β-carotene and a reference dose of labeled retinyl acetate. As we analyzed the data, the disposition of the two plasma isotope response curves led us to hypothesize that β-carotene bioefficacy relative to the reference dose could be estimated from the ratio of the two isotopes in a single blood sample, analogous to the plasma dual isotope ratio method for determining cholesterol absorption that was developed and tested by Zilversmit (7) and adapted by Green and Green for vitamin A absorption (8). In fact, mean β-carotene relative bioefficacy calculated from the plasma retinol isotope ratio (RIR^6) on d 2 was 12.5%, close to the values determined by modeling (13.5%) or by graphical methods based on areas under the plasma retinol tracer response curves ("area under the curve;" AUC_p) (13.1%) (6). We found that similar plasma retinol ratio methods for estimating bioefficacy had been previously postulated by Hickenbottom at al. (9) and Van Loo-Bouwman et al. (10), but the approach has apparently not received additional attention.

Here, we further investigate the plasma retinol isotope ratio for measuring β-carotene relative bioefficacy. We used model-based compartmental analysis (11) and the Simulation, Analysis and Modeling software (12) to verify the accuracy of the technique by first developing and then applying a compartmental model that would predict assigned values for β-carotene bioefficacy over a physiologically-reasonable range (5). We confirmed that our physiologically-based model predicted these assigned bioefficacies and then used the model to generate corresponding plasma isotope response data. Finally, we used the simulated plasma data to calculate relative bioefficacy by the plasma RIR and the isotope reference method [IRM (13)] and compared predictions to the known values. The IRM, in which the ratio of areas under the plasma isotope response curves after subjects ingest doses of labeled β-carotene and retinyl acetate, has been previously used to estimate β-carotene relative bioefficacy by several investigators, as reviewed by Tang (13). In addition to providing a way to evaluate the accuracy of the two methods, our model development and interpretation also provided insights into the complex processes and time course of the bioconversion of β-carotene to retinol, including its absorptive (intestinal) and post-absorptive (extra-intestinal) components. We show that the plasma retinol isotope ratio provides a simple, accurate, and easily-used method for estimating β-carotene relative bioefficacy.

Methods

Subjects. We selected ten subjects (see next paragraph) from previously-published studies in which participants received oral doses of stable isotope-labeled β-carotene and a labeled retinyl acetate reference dose. In one case (14), the reference dose was ingested 3 d after the dose of labeled β-carotene; plasma retinol tracer responses were measured for 56 (β-carotene-derived retinol) and 53 d (for retinyl acetate-derived retinol); β-carotene relative bioefficacy was estimated using the IRM. Subsequently, data for labeled plasma retinol derived from the retinyl acetate dose were modeled by Cifelli et al. (15) using WinSAAM v. 3.3.0 (12) to determine kinetic

parameters for retinol metabolism. In the second case (16), subjects ingested simultaneous oral doses of labeled β-carotene and retinyl acetate; plasma tracer responses were measured for 14 d; Green et al. (6) retrospectively modeled plasma retinol data for the two labels and used WinSAAM to estimate β-carotene relative bioefficacy for subjects whose results indicated little to no post-absorptive β-carotene bioconversion.

For the current work, our sample (n=10) includes individuals with a wide range of total body vitamin A stores (66 – 2500 μmol; **Supplemental Table 1**) and known vitamin A kinetics. We selected six subjects from the study of Cifelli et al. (15) [including the ones from Tang et al. (14) reported to have the lowest, average, and highest conversion of β-carotene] plus four subjects from Green et al. (6). Simulated data based on both published (6, 15) and assigned kinetic parameters for these individuals were used to advance understanding of β-carotene bioconversion and to evaluate the accuracy of the IRM and the newly-described plasma RIR for estimating β-carotene relative bioefficacy. We deemed ten to be an adequate number of subjects because it allowed for inclusion of a wide range of bioefficacies with differing contributions from absorptive and early/late post-absorptive bioconversion. Since we needed to have known values for bioefficacy in order to test the accuracy of predictions by the two methods, we assigned known, physiologically-reasonable values for total bioefficacy, including the absorptive and post-absorptive components, to each subject (see later).

Compartmental models. We used the six-compartment model presented by Green et al. (6) (**Figure 1A**) to describe the metabolism of retinyl acetate-derived retinol. In Figure 1A, components 1 to 4 represent the processing of dietary preformed vitamin A until it reaches the liver and is secreted into plasma compartment 5 as retinol bound to retinol-binding protein; retinol in the plasma pool exchanges with vitamin A in the storage pool (compartment 6), which is also the site of irreversible loss from the system.

Next, we developed a partially-parallel model (Figure 1B) that describes trafficking of labeled retinol derived from the conversion of absorbed β-carotene during both absorptive (intestinal) and early plus late post-absorptive (extra-intestinal) phases. A "partially-parallel model" means that we used the same values for kinetic parameters in the model for β-carotene-derived retinol as we used in the model for retinyl acetate-derived retinol when we were describing biochemical and physiological processes that one logically assumes to be identical for the two labels. It should be emphasized that this work focuses on retinol derived from the labeled β-carotene dose (i.e., on β-carotene that is converted to retinol and secreted into plasma after conversion). For the absorptive / intestinal pathway (Figure 1B, unshaded components), the model was parallel to the one in Figure 1A; that model had been developed (6) for subjects whose data indicated little to no post-absorptive conversion of absorbed β-carotene to retinol. For the post-absorptive / extra-intestinal pathway (Figure 1B, shaded components), we used aspects of the model presented by Novotny et al. (17) as well as other information in the literature (18-20). In Figure 1B, components 11 to 13 represent the processes of digestion and absorption of β-carotene; compartment 11 is the site of splitting of the β-carotene dose into the fraction eventually converted to retinol (that which is transferred to compartment 12) versus that which does not contribute to bioefficacy. Bioefficacy was partitioned into absorptive and post-absorptive components at delay component 13 as follows. For the intestinal bioconversion pathway, absorbed β-carotene is converted to retinol and packaged in chylomicrons, and the retinol (primarily retinyl esters) is transferred to compartment 14. For the post-absorptive pathway, chylomicron β-carotene is transferred from component 13 to compartment 21, which represents tissue uptake (both extrahepatic and hepatic) of β-carotene from chylomicrons or chylomicron remnants. This β-carotene can be converted to retinol in tissues (compartment 24), representing early post-absorptive conversion (solid-line arrow), or it may be delayed by passing from compartment 21 through components 22 and 23 (possibly plasma lipoproteins or adipocyte lipid droplets; see Discussion) before conversion to retinol and passage into compartment 24,

representing late post-absorptive conversion (dashed-line arrows). Similar to the models developed by Green et al. (21) and Novotny et al. (17), retinol in compartment 24 is then either secreted directly into plasma compartment 15 bound to retinol-binding protein or esterified for storage in compartment 16.

Assignment of known values for β-carotene bioefficacy, including both absorptive and post-absorptive components. In order to obtain known reference values for bioefficacy against which we could evaluate the RIR and IRM, we chose ten values for β-carotene relative bioefficacy ranging from 10 to 50%, reflecting the variability reported in the literature (5); then we assigned a value to each subject. Assignments were made based on individual total body vitamin A stores (Supplemental Table 1); the results of Green et al. (6), which indicated that the subjects in that study had little post-absorptive conversion; and the results of Tang et al. (14), from which we selected the three subjects that were reported to be the lowest, average, and highest converters of β-carotene. Each level of relative bioefficacy was then partitioned into hypothetical but physiologically-reasonable values for absorptive (7 – 40%) and post-absorptive (3 – 20%) components. For example, for subject 1, we assigned a total relative bioefficacy of 10% and attributed 7% bioefficacy to intestinal bioconversion and 3% to extra-intestinal processes. Additionally, each value for post-absorptive conversion was partitioned into early and late components (e.g., 2.4% early and 0.6% late for subject 1). As described in **Supplemental Methods 1**, we simulated the model for each individual to verify that it accurately predicted the assigned values for the various components of bioefficacy.

Use of compartmental analysis to generate plasma isotope data for retinyl acetate- and β-carotene-derived retinol. To generate plasma retinol tracer response data [fraction of dose in plasma (FD_p) versus time] for the retinyl acetate reference dose, we used the kinetic parameters

157 reported for the ten subjects (Supplemental Table 1), including an absorption efficiency of 75%,
158 as inputs in the compartmental model presented by Green et al. (6) (Figure 1A). Time points
159 (n=21; 0, 3, 6, 9, and 11 h, then daily for 9 d, and weekly from 14 to 56 d) for the simulation were
160 based on the study of Tang et al. (14), in which extensive blood sampling had been done to
161 provide the detailed plasma tracer kinetic curves needed for model-based compartmental
162 analysis.

163 Next, to generate corresponding data for β-carotene-derived retinol, we fixed parameters
164 in the model shown in Figure 1B at the values presented in Supplemental Table 1. These
165 included model parameters described in **Supplemental Methods 2** that generated the assigned
166 values for total, absorptive, and post-absorptive (early and late) bioefficacy. Then, data for FD_p
167 versus time (3 h to 56 d) were simulated to generate plasma tracer response profiles for retinol
168 derived from the β-carotene dose for each of the ten subjects at the various levels of bioefficacy.
169 For each subject, simulations resulted in plasma data for labeled retinol derived from three
170 sources: the retinyl acetate reference dose, absorptive (intestinal) conversion of β-carotene to
171 retinol, and post-absorptive (extra-intestinal, early and late) conversion of β-carotene to retinol.
172 Datasets for FD_p versus time were used to calculate β-carotene relative bioefficacies based on
173 plasma isotope ratios and areas under the curves as described in the next sections.
174
175 *Calculation of β-carotene relative bioefficacy from the plasma retinol isotope ratio.* Relative
176 bioefficacy was calculated for the datasets described above based on retinol isotope ratios in
177 plasma at specific times using Equation *1*:
178
179 RIR relative bioefficacy (%) = (FD_p for labeled β-carotene-derived retinol /
180 FD_p for labeled retinyl acetate-derived retinol) × 100 (*1*)
181

The plasma RIR was calculated using FD_p data in molar retinol activity equivalents (1 μmol β-carotene = 2 μmol retinol) at times from 1 to 56 d after dose administration.

Calculation of β-carotene relative bioefficacy using the isotope reference method. Relative bioefficacy was calculated for the same data using the IRM as applied by Tang et al. (14) and Green et al. (6) as shown in Equation *2*:

$$\text{IRM relative bioefficacy (\%)} = (AUC_p \text{ for labeled β-carotene-derived retinol} / AUC_p \text{ for labeled retinyl acetate-derived retinol}) \times 100 \quad (2)$$

The trapezoid rule was applied to FD_p data using GraphPad Prism v. 7.0 for Windows (GraphPad Software Inc., La Jolla, CA) to determine AUC_p from time zero to the end of the experiment (56 d) or to earlier times.

Comparison of calculated values for relative bioefficacy to known values. β-Carotene bioefficacies calculated for each individual using the plasma RIR (Equation *1*) and the IRM (Equation *2*) were compared to the known (assigned) values for total, absorptive, and post-absorptive (both early and late) bioefficacy in order to evaluate the accuracy of the two techniques.

Application of the plasma retinol isotope ratio and the isotope reference method to previously-published kinetic data. We expanded the application of the plasma RIR (Equation *1*) and IRM (Equation *2*) to estimate β-carotene relative bioefficacy for the group of healthy young adults (n=30) that had been previously studied by Green et al. (6). In that paper, relative bioefficacies were calculated using the model-based AUC_p method in WinSAAM [i.e., (plasma residence time for β-carotene-derived retinol divided by plasma residence time for retinyl acetate-derived retinol)

x 100]. These values were compared in (6) to estimates calculated using the IRM at 14 d; results for the plasma RIR at d 2 were also mentioned in (6). For the current analysis, relative bioefficacy was calculated using Equations *1* and *2* at 1, 2, 7, and 14 d.

Statistical analysis. Figures were prepared and linear regression analysis was done using GraphPad Prism v. 7.0 for Windows. To identify time-dependent changes in bioefficacy calculated using the RIR and IRM for subjects from (6), we used repeated measures ANOVA (JMP® Pro v. 12.1.0; SAS Institute Inc., Cary, NC) and to assess within-subject differences in predictions, we used matched pairs analysis (JMP® Pro v. 12.1.0). $P < 0.05$ was considered significant.

Results

Confirmation of assigned (known) values for β-carotene bioefficacy. We used model simulations as described in Supplemental Methods 1 to confirm that our physiologically-based compartmental model (Figure 1B) accurately predicted the assigned values for the various components of bioefficacy (total, absorptive, and early and late post-absorptive) for each individual. As shown in **Figure 2** for subject 5, the simulation correctly predicted the assigned values for total (actual, not relative) bioefficacy (18.75%), absorptive bioefficacy (15%), and post-absorptive bioefficacy (3.75%), as well as the components of post-absorptive (early at 1.69% and late at 2.06%). For this subject, values for all components plateaued by 5 d and were time invariant after that. Model simulations also precisely predicted assigned (known) values for bioefficacy, including all forms of bioconversion, for the other nine subjects (data not shown).

Plasma isotope response data/curves for the retinol reference dose and for β-carotene-derived retinol. Using the model shown in Figure 1A and the kinetic parameters from Cifelli et al. (15) and Green et al. (6) that are presented in Supplemental Table 1, we simulated tracer data for plasma

retinol derived from the retinyl acetate reference dose versus time. As shown in **Figures 3A** and **B** (solid lines) for subjects 2 and 8, respectively, plasma retinol tracer peaked ~13 h after dosing and then gradually fell as retinol was distributed to tissues. The bend in the curves at ~7 d indicates recycling of tracer back to plasma. By 10 d post-dosing, the curves entered a terminal slope, equivalent to the apparent system fractional catabolic rate (~3%/d for these two subjects). Similar patterns were observed for the retinyl acetate-derived retinol curves for the other subjects (data not shown).

Also presented in Figures 3A and B are the simulated plasma tracer response data for β-carotene-derived retinol (dashed lines) when relative bioefficacy was fixed at 14% (10% absorptive and 4% post-absorptive; subject 2) or at 40% (30% absorptive and 10% post-absorptive; subject 8). Note that, at the higher (versus lower) level of total β-carotene bioefficacy, the curve for β-carotene-derived retinol is closer to the one for retinol from the reference dose. For all subjects (data not shown), before any late post-absorptive conversion occurred, curves for β-carotene-derived retinol were similar to those for retinyl acetate-derived retinol (i.e., the two curves appeared to be parallel, as illustrated in Figures 3A and B). At the time set for late post-absorptive conversion (delay component 22; Figure 1B), which was varied from 2 to 5 d across subjects (Supplemental Table 1), there was an increased influx of tracer into plasma as newly-formed labeled retinol was secreted into compartment 15. At the higher levels of post-absorptive conversion (7 to 20%), the increased input was evident as a discernible hump in the curves for β-carotene-derived retinol (as illustrated by the data in Figure 3B), whereas at the lower levels of post-absorptive conversion (3 to 5%), this influx caused only subtle changes in the curves (as in Figure 3A). Plasma retinol tracer stabilized after late post-absorptive conversion and the curves entered a terminal slope by ~10 d, becoming parallel to the curve for retinyl acetate-derived retinol.

Next, to examine the two components of bioefficacy separately, we used model-based compartmental analysis to simulate the expected plasma isotope response curves for β-carotene-

derived retinol formed from absorptive versus post-absorptive conversion. **Figure 4** shows these simulations, along with the curve for retinol derived from the reference dose, for subject 5; see also **Supplemental WinSAAM Deck**. As was the case for all subjects (data not shown), the curve for absorptive bioconversion (dash-dot line in Figure 4) peaked at ~13 h, similar to that for the retinyl acetate-derived retinol (solid line); curves were parallel from this point on. The curve for post-absorptive conversion (dotted line) showed two distinct peaks. The first peak occurred ~13 h after dosing, similar to that for the absorptive phase, representing early post-absorptive conversion, whereas the second peak occurred after late post-absorptive conversion (2.5 d). Our model simulation predicts that a portion of the retinol formed during early post-absorptive conversion enters plasma with the same time course as intestinally-produced retinol; see Discussion. When there is extensive late post-absorptive conversion, there is a hump in the composite curve for β-carotene-derived retinol (absorptive plus post-absorptive; dashed line). After ~10 d, the curves for β-carotene-derived retinol parallel that for the retinyl acetate-derived retinol for the duration of the experiment (56 d).

β-Carotene relative bioefficacy calculated by the RIR and IRM. Relative bioefficacies predicted by the plasma RIR (Equation *1*) and the IRM (Equation *2*) were compared to known (assigned) values for total, absorptive, and post-absorptive relative bioefficacy for the ten subjects (**Table 1**). For all subjects, the plasma RIR accurately predicted known values for total relative bioefficacy from 14 d to the end of the experiment (56 d); specifically, the RIR at either 14 or 56 d predicted a mean of 101% of the known values (range, 100 to 103%). Bioefficacy estimates calculated by the IRM predicted a mean of 92% (range, 74 to 99%) and 95% (range, 84 to 100%) of total bioefficacy at 14 and 56 d, respectively. For 8 of the 10 subjects, the IRM predictions were very close to the known values (96 and 98% of the total at 14 and 56 d, respectively)

We then compared the equations' predictions on d 1 to known values for absorptive bioefficacy, as has been done in earlier applications of the IRM when the prediction on 1 d has

been said to indicate intestinal bioconversion (14, 22). The d 1 plasma RIR overestimated the assigned values for absorptive bioconversion by a mean of 17% and the IRM overestimated them by 26% (Table 1), suggesting that the d 1 predictions included more than absorptive conversion. As shown in Figure 4 inset, at 1 d, the composite curve for β-carotene-derived retinol (dashed line) is higher than the curve for retinol derived from intestinal conversion (dash-dot line). This difference is attributable to a contribution from early post-absorptive conversion (see Discussion). Note that, when the plasma RIR and IRM were calculated using the appropriate simulated data for retinol derived from intestinal β-carotene conversion and the retinyl acetate reference dose, both methods predicted known values for absorptive bioefficacy at all times from 1 d on (data not shown). When known values for absorptive and early post-absorptive bioefficacy were added together using data in Table 1, the RIR at 1 d predicted a mean of 96% of the sum (range, 85 to 103%); the linear regression equation was $y = 0.95x + 1.8$ ($R^2 = 0.99$), where y is the known value for absorptive plus early post-absorptive bioefficacy and x is the RIR on d 1. This indicates that, on average, ~4% of the retinol produced by early post-absorptive conversion appeared in plasma after d 1 and is included in the prediction of bioefficacy at 14 d. For the IRM, calculations on d 1 predicted 100% (range, 79 to 120%) of the sum of the absorptive plus early post-absorptive bioefficacy. Thus, in contrast to previous assumptions that d 1 predictions by the IRM represent absorptive bioconversion (14), our model predicts that d 1 data include not only the absorptive but also some of the early post-absorptive conversion of β-carotene to retinol.

To illustrate the changes in relative bioefficacy calculated by the RIR and IRM over time, we present in **Figure 5** results for subject 7 who was assigned 35% total relative bioefficacy, partitioned into 28% absorptive and 7% post-absorptive (2.1% early and 4.9% late). For this subject, the plasma RIR at 1 d predicted a relative bioefficacy of 30%, close to the known value for absorptive plus early post-absorptive bioefficacy (30.1%). After late post-absorptive conversion occurred (4.5 d for subject 7) and newly converted retinol derived from β-carotene entered plasma, the RIR was higher (i.e., the numerator in Equation *1* increased), peaking at 6.5

d at 48% (138% of the total). The value then fell and stabilized by 14 d at 35.5%, near the known value for total relative bioefficacy (35%). For all subjects, the peak in the RIR occurred between 4 and 7 d post-dosing; by 14 d, the RIR stabilized near the known value for total relative bioefficacy. Similar trends versus time were observed for the other subjects (data not shown). These results show that the RIR is highly responsive to late post-absorptive conversion and needs time to stabilize; for these subjects, when late conversion occurred between 2 and 5 d, stabilization in the ratio was evident by 14 d.

In the case of the IRM, predictions for subject 7 (Figure 5) gradually increased following late post-absorptive conversion, averaging 92 and 95% of the total bioefficacy at 14 and 56 d, respectively. Similar trends were observed for the other subjects (data not shown). Overall, the IRM predictions at 14 or 56 d were close to the assigned values for total relative bioefficacy, except when there was substantial (late) post-absorptive conversion. Specifically, at the two highest levels of late post-absorptive bioconversion (subjects 6 and 10; Table 1), the IRM underestimated total relative bioefficacy by ~25% at 14 d and ~15% at 56 d.

Comparison of predictions of the plasma RIR and the IRM when applied to previously-published retinol kinetic and bioefficacy data. We applied Equations *1* (RIR) and *2* (IRM) to previously-published data (6) for 30 adults for whom bioefficacy had been estimated using WinSAAM. Mean values for relative bioefficacy predicted by the RIR and IRM are plotted over time in **Figure 6**. Predictions of the two equations were similar at 1 and 2 d (~12%). For the plasma RIR, predictions were significantly higher on d 7 and d 14 (~15%) compared to d 1 and 2, indicating that some post-absorptive conversion of β-carotene to retinol (~3%) occurred between 2 and 7 d in some of these subjects, even though plasma isotope response curves for subjects in (6) suggested little to no post-absorptive conversion. Based on our theoretical work, we hypothesize that predictions of relative bioefficacy by the RIR at 1 and 2 d include all of the absorptive and most of the early post-absorptive bioconversion, and estimates at 7 and 14 d include the late

post-absorptive conversion as well. For the IRM, predictions were higher on 7 d versus d 1 and 2, and higher still on 14 d (~13%), also suggesting some post-absorptive bioconversion (~1%). For individual comparisons, the RIR on d 14 predicted significantly higher values for relative bioefficacy compared to the IRM, by a mean difference of 2.3% bioefficacy ($P < 0.0001$). Relative bioefficacy predicted by the RIR on d 14 ranged from 4.6 to 37.1% over all subjects, whereas for the IRM, 14 d predictions ranged from 4.5 to 30.7%. The maximum difference in relative bioefficacy values at 14 d versus 1 d was 7.7% for the RIR and 5.1% for the IRM. These results suggest that the RIR is more responsive than the IRM to (late) post-absorptive bioconversion, even when little occurs. Based on our theoretical analysis, we suggest that the RIR likely provides a more accurate estimate of β-carotene relative bioefficacy using data from a single blood sample obtained at 14 d or later.

Discussion

In this paper, we show (Table 1) that β-carotene relative bioefficacy is accurately estimated based on the ratio of two retinol isotopes in a single plasma sample (Equation *1*) obtained at 14 or more days after co-ingestion of labeled β-carotene and a labeled retinyl acetate reference dose. Such an approach had been previously suggested by Hickenbottom et al. (9) and Green et al. (6) but had not been evaluated or used. The single sample approach will make it more feasible to measure β-carotene relative bioefficacy in field settings, where it can be difficult to obtain multiple samples over time, as is done when using the isotope reference method (Equation *2*) to estimate relative bioefficacy (13). In addition, the single sample method will facilitate measuring bioefficacy in children, in whom repeated sampling is difficult. It is worth noting that investigators could also assess vitamin A status by collecting an additional blood sample at 4 or 5 d and applying an appropriate retinol isotope dilution equation to the retinyl acetate-derived retinol data to predict individual vitamin A total body stores (23).

To our knowledge, this study is the first to evaluate the accuracy of methods for estimating β-carotene relative bioefficacy. We used model-based compartmental analysis (11) and model simulations to verify predictions of known (assigned) values for bioefficacy (Figure 2) and then applied Equations *1* and 2 to calculate relative bioefficacy using simulated plasma retinol tracer response data. This approach is similar to the one we recently used to test the accuracy of an equation for estimating vitamin A total body stores at isotopic equilibrium (24). Here, in addition to demonstrating that the plasma RIR accurately predicts β-carotene relative bioefficacy at 14 d or later after dosing, we also showed that the IRM accurately predicts relative bioefficacy for some subjects (specifically, those with low levels of late post-absorptive bioconversion). It would be interesting for other investigators to now apply the RIR retrospectively to their previously-collected data from IRM studies to compare results for the two methods.

It should be emphasized that both the plasma RIR and IRM estimate bioefficacy *relative* to the absorption efficiency of the co-administered retinyl acetate reference dose. As methods are developed for measuring vitamin A absorption efficiency, it will be possible to correct estimates of β-carotene relative bioefficacy by including the actual value for absorption efficiency of the reference dose (bioefficacy = relative bioefficacy × reference dose absorption efficiency).

In addition to providing a way to evaluate the accuracy of methods for estimating β-carotene relative bioefficacy, compartmental modeling also led to insights into the time course and likely pathways for conversion of absorbed β-carotene to retinol. For provitamin A, previous work (14, 17, 22, 25) has suggested that some of the absorbed carotenoid is converted to retinol in the intestine and another portion is converted later (post-absorptive bioconversion) in tissues such as liver and adipose tissue. Starting with these assumptions, the model (Figure 1B) developed to generate plasma isotope response data for retinol derived from β-carotene included a site (delay component 13) for partitioning absorbed β-carotene destined for intestinal conversion (compartment 14) or for post-absorptive conversion (compartment 21). We hypothesize that β-carotene in chylomicrons taken up by the liver or extrahepatic tissues could be

converted to retinol (compartment 24) soon after uptake, representing the early post-absorptive phase. Alternatively, β-carotene could be transferred from compartment 21 to delay component 22 and compartment 23, representing the late post-absorptive conversion pathway. The late post-absorptive route might involve β-carotene incorporation into very low density lipoproteins in the liver, with release of those lipoproteins into the circulation and subsequent metabolism to low- and high density lipoproteins (delay component 22), followed by uptake of plasma lipoprotein β-carotene by tissues in which post-absorptive bioconversion can occur. Alternatively, the late post-absorptive route might involve uptake of chylomicron β-carotene into (for example) adipose tissue and then partitioning into lipid droplets with subsequent conversion to retinol (compartment 24). As shown in Figure 1B, this retinol can either be secreted into plasma compartment 15 bound to retinol-binding protein or it can be esterified for storage in compartment 16.

Plasma isotope response curves like the one for β-carotene-derived retinol in Figure 3 reflect the composite impact on retinol kinetics of all β-carotene bioconversion processes that result in the appearance of labeled retinol in plasma; methods like the RIR and IRM estimate relative bioefficacy using plasma retinol tracer data. Thus, if some absorbed β-carotene is converted locally in tissues to biologically active forms of vitamin A (26) or to apocarotenoids (27), those compounds never reach plasma as retinol and so these quantitatively minor pathways do not contribute to bioefficacy as currently defined (2).

As illustrated in Figure 4, one can use model simulations to look at the likely time course of the individual components of bioefficacy. This analysis revealed two distinct peaks in the curve for retinol derived from post-absorptive conversion: the first, corresponding to early post-absorptive conversion, occurs at the same time (~13 h after dose ingestion) as the peak in the curve for retinol generated by absorptive bioconversion; the second peak (at 2.5 d) corresponds to the late post-absorptive process. Depending on the extent of late post-absorptive conversion, there may be a discernible “hump” in the composite curve for β-carotene-derived retinol

(absorptive plus post-absorptive). This is evident in Figure 3B and can be seen in the results of other investigators (14, 17, 28).

Our current results suggest that retinol formed from early post-absorptive conversion enters plasma with the same time course as intestinally-produced retinol. Thus, based on our model, predictions of the plasma RIR at 1 d include retinol derived from absorptive conversion and some (probably most) of the early post-absorptive conversion; the early post-absorptive component might include some conversion of the labeled β-carotene dose that occurs in the intestine following subsequent meals. In earlier applications of the isotope reference method (14, 22), d 1 results have been interpreted to represent intestinal bioconversion. Similar to results by Tang et al. (14) which indicated that 81% of the total β-carotene conversion to vitamin A had occurred by 1 d, our results indicate that for the RIR, 81% of the total relative bioefficacy was predicted at 1 d (86% for the IRM). By collecting a blood sample at 1 d as well as 14 d, researchers could use the RIR to estimate the contribution of absorptive- and early post-absorptive conversion to total relative bioefficacy.

Our data indicate that, after late post-absorptive conversion, the RIR transiently overestimates relative bioefficacy before stabilizing at the correct value for “total” bioefficacy by 14 d. Time for ratio stabilization is also required for the plasma dual isotope ratio method used to determine cholesterol absorption (7) and vitamin A absorption (8). It is interesting to note that, if RIR predictions on d 14 for a given individual are substantially higher than those on d 1, this indicates that late post-absorptive bioconversion is an important contributor to total bioefficacy in that subject.

In conclusion, compared to graphical methods (e.g., the isotope reference method), the plasma retinol isotope ratio provides a simpler and more accurate method for estimating carotenoid relative bioefficacy following co-administration of isotope-labeled doses of β-carotene and retinyl acetate (reference dose). The plasma retinol isotope ratio method should be valid for use in both children and adults, and it can be used for test solutions (e.g., isotopes administered

in oil) or for intrinsically-labeled carotenoid in foods. The plasma RIR predicts β-carotene relative bioefficacy based on analysis of one blood sample collected 14 d or more after dosing.

Acknowledgments

The human studies done at Newcastle University [cited as references (6) and (16)] were supported by the Biotechnology and Biological Science Research Council, U.K. (grant BB/G004056/1). J.L.F. and M.H.G. designed and conducted the research described and analyzed the data; G.L. and A.O. helped refine the model and provided an essential dataset; and J.L.F., J.B.G., and M.H.G. wrote the paper. M.H.G. has primary responsibility for the content presented here. All authors read and approved the final manuscript.

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TABLE 1 Assigned (known) and predicted values for β-carotene relative bioefficacy (%) for ten subjects[1]

Subject	1	2	3	4	5	6	7	8	9	10
Assigned values										
Total	10	14	18	21	25	30	35	40	45	50
Absorptive	7	10	9	16	20	15	28	30	40	30
Post-absorptive	3	4	9	5	5	15	7	10	5	20
Early	2.4	3	6.75	4.25	2.25	5.25	2.1	2	2.5	2
Late	0.6	1	2.25	0.75	2.75	9.75	4.9	8	2.5	18
Values predicted by RIR										
1 d	8.1	12.9	13.6	19.6	23.0	17.2	29.8	32.7	41.8	31.5
2 d	8.3	11.0	12.0	20.5	22.0	17.1	29.7	32.7	40.8	31.0
4 d	11.2	15.6	23.2	20.6	32.1	40.8	29.3	34.4	41.1	31.0
7 d	10.0	14.2	18.1	22.5	27.3	31.5	47.8	69.2	49.2	62.9
14 d	10.0	14.0	18.1	21.1	25.2	30.4	35.5	40.9	45.1	51.3
21 d	10.0	14.0	18.1	21.1	25.2	30.4	35.4	40.8	45.1	51.4
28 d	10.0	14.0	18.1	21.1	25.2	30.4	35.4	40.8	45.1	51.4
56 d	10.0	14.0	18.1	21.1	25.2	30.4	35.4	40.8	45.1	51.4

Values predicted by IRM										
1 d	10.1	15.6	18.3	20.2	23.3	16.0	27.2	30.1	42.7	29.9
2 d	9.2	13.8	15.6	20.0	23.0	16.6	28.7	31.6	42.1	30.6
4 d	9.2	13.6	15.2	20.1	23.1	17.4	29.0	31.9	41.7	30.7
7 d	9.4	13.9	16.3	20.7	24.5	20.0	30.5	36.8	42.5	36.3
14 d	9.6	13.9	16.8	20.8	24.7	22.1	32.2	38.7	42.9	38.4
21 d	9.7	13.9	17.0	20.8	24.8	23.4	32.6	38.9	43.1	39.5
28 d	9.8	13.9	17.1	20.9	24.8	24.2	32.8	39.1	43.3	40.4
56 d	9.8	14.0	17.3	20.9	24.9	25.8	33.3	39.4	43.7	42.2

[1] Values are percent β-carotene relative bioefficacy for 10 subjects selected from previously published studies (6, 15). The top section shows values for relative bioefficacy [total, absorptive, and post-absorptive (early and late)] that were assigned to each subject as described in Methods. The middle section shows predictions of the plasma retinol isotope ratio [RIR; Equation *1* (see Methods)] versus time after co-ingestion of an oral dose of stable isotope-labeled β-carotene and a retinyl acetate reference dose; the bottom section shows predictions of the isotope reference method [IRM; Equation *2* (see Methods)] versus time after dosing.

Figure Legends

1. Proposed compartmental models for retinol kinetics in humans following co-ingestion of labeled β-carotene and labeled retinyl acetate. Circles represent compartments, rectangles correspond to delay components, interconnectivities between compartments [L(I,J)s] are fractional transfer coefficients or the fraction of retinol or β-carotene molar retinol activity equivalents transferred to compartment I from compartment J each day, and delay times [DT(I)] correspond to the time spent in delay component I. The asterisks represent the site of input of the orally administered tracers; U(1) and U(11) represent dietary vitamin A and β-carotene input, respectively; and the triangles represent the site of sampling (plasma). Panel A shows the six-compartment model adapted from (6) that describes the metabolism of retinyl acetate-derived labeled retinol. Compartments 1 to 4 represent the processing of dietary preformed vitamin A; fractional absorption of retinyl acetate was set at 0.75. Diet-derived vitamin A is taken up by hepatocytes (compartment 4), primarily as retinyl esters, and then retinol is secreted into plasma compartment 5 bound to retinol-binding protein; retinol in the plasma pool exchanges with vitamin A in the extravascular storage pool (compartment 6), which is the site of irreversible loss from the system. Panel B shows the ten-compartment model developed to describe the metabolism of β-carotene-derived retinol during absorptive (intestinal) conversion (unshaded components) as well as early and late post-absorptive (extra-intestinal) conversion (shaded components). Components 11 to 13 represent gastrointestinal transit and absorption of β-carotene; in compartment 11, absorbed β-carotene is partitioned into the fraction eventually converted to retinol (that which is transferred to compartment 12) versus that which is not converted [(L0,11)], and in delay component 13, bioefficacy is partitioned into absorptive and post-absorptive components. For the absorptive pathway, retinol (primarily as retinyl esters) derived from absorbed β-carotene is transferred to compartment 14 and processed as described above in compartments 15 and 16. For the post-absorptive pathway, absorbed β-carotene is

transferred from component 13 to compartment 21, representing uptake by hepatic and extrahepatic tissues. For the early post-absorptive pathway (solid-line arrow), some of this β-carotene in compartment 21 is converted in situ to retinol (compartment 24). For the late post-absorptive pathway (dashed-line arrows), the β-carotene passes from compartment 21 through component 22 and compartment 23 before entering compartment 24. Retinol in compartment 24 can either be secreted directly into plasma (compartment 15) or esterified for storage (compartment 16). βC, β-carotene; ROH, retinol; RE, retinyl esters.

2. Model simulations for fraction of the β-carotene dose in various compartments versus time for subject 5. Shown are fraction of dose (as molar retinol activity equivalents) in model compartments (Figure 1B) that were designated as sinks (see Supplemental Methods 1) for total (compartment 12), absorptive (compartment 14), and post-absorptive bioefficacy (compartment 21), as well as for the early (compartment 24 with no input from compartment 23) and late post-absorptive components (compartment 23). Assigned values for bioefficacy (%) are indicated at each curve's plateau.
3. Model-predicted plasma retinol response curves versus time after co-ingestion of labeled β-carotene and labeled retinyl acetate for two subjects. Shown are simulated data for fraction of dose in molar retinol activity equivalents over time for labeled retinol derived from the retinyl acetate reference dose and from the β-carotene dose. Panel A shows simulations for subject 2 (Table 1) who was assigned values of 14% total relative bioefficacy (10% absorptive plus 4% post-absorptive bioconversion) with late post-absorptive conversion set at 2 d; panel B shows the corresponding simulations for subject 8 (40% total, of which 30% was absorptive and 10% post-absorptive); late post-absorptive conversion was set at 3.5 d.
4. Model-predicted plasma retinol response curves versus time after co-ingestion of labeled β-carotene and labeled retinyl acetate for subject 5. Shown are simulated values for fraction of dose in molar retinol activity equivalents over time (shown on a semi-log scale) for labeled retinol derived from the retinyl acetate reference dose and for β-carotene-derived retinol, as

well as retinol formed from absorptive and post-absorptive conversion of β-carotene. Subject 5 (Table 1) was assigned 25% total relative bioefficacy (20% absorptive plus 5% post-absorptive, with 2.25% early post-absorptive and 2.75% late post-absorptive). The inset shows these simulations for β-carotene-derived retinol on an expanded (linear) scale between 3 h and 10 d.

5. β-Carotene relative bioefficacy calculated by two methods. The plasma retinol isotope ratio (RIR; see Methods, Equation *1*) and the isotope reference method (IRM; Equation *2*) were used to calculate β-carotene relative bioefficacy over time for subject 7 (Table 1) who was assigned a value of 35% total relative bioefficacy (28% absorptive plus 7% post-absorptive); late post-absorptive conversion was set at 4.5 d post-dosing.
6. β-Carotene relative bioefficacy predicted by two methods for previously-studied adults (6). Values are means ± SEM (n=30) for relative bioefficacy calculated at four times after co-ingestion of labeled β-carotene and a labeled retinyl acetate reference dose; values were calculated using the plasma retinol isotope ratio (RIR; see Methods, Equation *1*) and the isotope reference method (IRM; Equation *2*). Means within each method that have different letters are significantly different ($P < 0.05$) using repeated measures ANOVA.